THE IN VIVO STABILITY OF ANTIBODY*

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Our previous studies, concerned largely with antigen retention and the characterization of antigen in tissues, led us to suspect that some antibody, like antigen, may be stabilized in tissue sites (1). The purpose of the present investigation was to study further, such antibody which appeared to be bound with antigen and perhaps normal tissue constituents. The investigation was carried out in the following manner: rabbits were first immunized by a series of intravenous injections of antigen and at the height of precipitin production they were fed S^{35}-labelled yeast cells. Newly produced antibody, like the other plasma proteins, became readily labelled with S^{35} amino acids within a few hours. After varying lengths of time, when circulating antibody had declined, antigen was again injected. Serum samples were then taken at various intervals and the specific activity of antibody was measured as soon as antibody reappeared in the circulation. The specific activity of antibody was measured in the antigen-antibody precipitates obtained, both in the period when the antibody had been allowed to decline and also after antigen had been reinjected to induce an anamnestic response. A comparison of antibody-specific activity provided evidence that there was a release of antibody which had been made at the time of S^{35} feeding and stored in some stabilized form in tissues. The results are discussed with respect to the anamnestic response and the retention of antigen in tissues.

Materials and Methods

Immunization.—Bovine serum albumin (Armour BSA) was the immunizing antigen except in one experiment in which a mixture of BSA and keyhole limpet hemocyanin (KLH)[1] was used. Intravenous injections were given on alternate days to adult rabbits weighing about 3 kg. When BSA was injected alone, the dose was 1 ml. of such concentration as indicated in individual experiments. When rabbits were immunized simultaneously with BSA and KLH, they were first given a few preliminary injections of BSA. This was necessary

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[1] KLH was prepared from the body fluid of keyhole limpets as described in reference 2.

in order to obtain antiserums, which contained approximately the same order of concentration of both species of antibody.

In order to label antibody being actively produced by rabbits, an injection of antigen was given intravenously, followed immediately by a feeding of yeast cells which had incorporated S^{35} into cell protein. Injection of antigen was stopped until a somewhat later time when a quantity of antigen was given which was sufficient to combine with most of the circulating antibody. After the removal of circulating antibody by the reinjection of antigen, bleedings were made at frequent intervals, several per day for the 2 to 3 days after the antigen injection, then at daily intervals for several days.

Determination of Antibody Nitrogen and Specific Activity of Antibody.—Rabbits had a circulating titer of several hundred gamma antibody nitrogen per ml. of serum, and were thus hyperimmune when an experiment was begun with a feeding of yeast cells and a reinjection of antigen. Serums, obtained from ear bleedings, were collected and stored in the frozen state. Thawed serums were clarified by centrifugation at 30,000 R.P.M. for 15 minutes, then absorbed twice with either 100 gamma pneumococcus SSSII precipitate or 100 gamma anti-KLH–KLH precipitate[2] per ml. serum in order to remove from the serums any substances which would absorb non-specifically in a subsequent test with the immunizing antigen. When serums required dilution for testing, the diluent was pooled normal rabbit serum, also clarified and absorbed by the same non-cross reacting antigen-antibody precipitate as used for the test serums. Specific precipitates were prepared by the addition of varying dilutions of the immunizing antigen to equal amounts of the absorbed antiserum. The precipitates were washed thoroughly after standing 48 hours at 4°C. and then analyzed by the quantitative precipitin method as described by Lanni and Campbell (3). After the point of maximal precipitation was determined by nitrogen assay, duplicate tubes were prepared with antigen and antibody corresponding to the amounts used to obtain the maximal precipitate. These latter precipitates were handled similarly as those for a nitrogen determination through the washing procedure, after which 2 to 3 drops of 0.1 N NaOH were used to dissolve the precipitate which was then transferred quantitatively with 2 ml. of water to an aluminum planchet. The solutions on the planchets were evaporated beneath a heat lamp and the residues were inserted in an end-window Geiger counter to determine the radioactivity. The determinations of radioactivity and of antibody nitrogen in the precipitate at the maximal point of precipitation were used to assign a specific activity to antibody as counts/minute/gamma of antibody N in 1 ml. serum.

Labelling of Yeast Cell Protein.—Yeast cells were grown in the medium defined by Williams and Dawson (4). The inoculum per liter of medium was ¼ cake commercial pressed yeast, to which 1.5 mc. S^{35} as sulfate was added. The culture was grown 40 hours at 30°C. with constant aeration by introduction of a vigorous stream of washed air through a coarse sintered glass disk. Cells were collected by centrifugation for 30 minutes at 3000 R.P.M. and 4°C. The medium was decanted and an aliquot counted. The cells were washed twice with saline; aliquots of both the cells and the combined washings were assayed for radioactivity in order to determine the distribution of radioactivity. There was about 80 per cent incorporation of S^{35} radioactivity into yeast cells. Yields were not increased by either a greater inoculum or a longer incubation. Rabbits received 3 feedings by gavage given as an equal quantity of cell suspension on consecutive days. The total amount of radioactivity so administered to an animal was 1.0 to 1.5 mc. Antigen was given always at the time of the first feeding but not during other feedings. Subsequent injection of antigen was made as indicated in the separate experiments.

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[2] Both the KLH and SSSII precipitates used for absorption were prepared with rabbit antiserum.

EXPERIMENTAL

Antigen Reinjected at 11 and 102 Days.—

Two rabbits (Nos. 6-93 and 6-97) received intravenously eight, 1 ml. injections of 4 per cent BSA. Thirteen days after the last injection, the animals were bled to obtain a few milliliters of serum for an antibody nitrogen determination. A few minutes later 1 ml. of 4 per cent BSA was injected, followed by a force feeding of 15 ml. of a suspension of yeast cells containing 6 ml. of packed cells with 0.35 mc. S^{35}. These events mark the 0 time to which

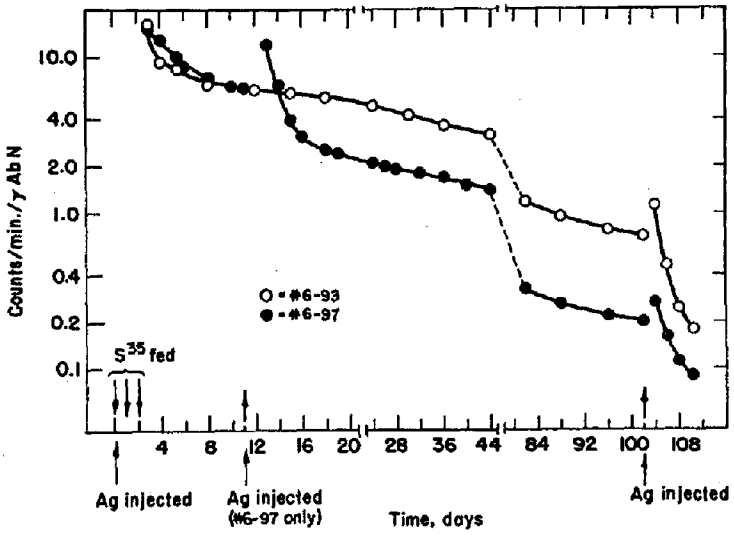

FIG. 1. The specific activity of circulating antibody in hyperimmune rabbits fed S^{35}-labelled yeast cells at days 0, 1, and 2. The last in a series of BSA injections was made simultaneously with the initial feeding of yeast cells at 0 time. Immediately after a bleeding on day 11, BSA was again injected into No. 6-97. The specific activity of No. 6-93, which was not reinjected, shows a normal decline for comparison with No. 6-97. At day 102, both Nos. 6-93 and 6-97 were again reinjected.

future manipulations were referred (Fig. 1). The same amount of yeast cell suspension was fed on each of the following 2 days. Bleedings were made at frequent time intervals to obtain serum. The serums were absorbed twice with precipitates of KLH–anti-KLH. After this absorption, the serums were tested with the immunizing antigen to determine the amount of specific antibody.

The injection of antigen was found to have removed circulating antibody as shown by negative interfacial ring tests of serum layered with antigen. At 3 days each animal had a titer exceeding 100 gamma antibody nitrogen per ml. of serum (Table I). The maximal titer occurred within 5 to 8 days after the antigen injection. On day 11 after the previous antigen injection, 1 animal (No. 6-97) again received an injection of 1 ml. 4 per cent BSA. Rabbit 6-93

TABLE I

Specific Activity of Antibody in the Serums of Nos. 6-93 and 6-97 before and after the Reinjection of BSA

Time	No. 6-93			No. 6-97		
	Ab N	S^{35}	Specific activity	Ab N	S^{35}	Specific activity
days						
0	310	0	0	82	0	0
3	165	2722	16.5	105	1628	15.5
4	470	4418	9.4	330	4290	13.0
5	650	5460	8.4	385	3850	10.0
6	—			500	4400	8.8
8	600	4080	6.8	470	3478	7.4
11	—			325	2112	6.5
Ag → inj.						
12	375	2325	6.2	0		
13	—			12	144	*12.0*
14	—			102	682	6.7
15	230	1357	5.9	165	660	4.0
16	—			225	698	3.1
18	188	1053	5.6	320	830	2.6
19	—			230	552	2.4
24	150	735	4.9	150	315	2.1
36	108	399	3.7	100	170	1.7
82	45	54	1.2	39	13	0.33
102	12	9	0.72	10	2	0.20
Ag → inj.						
104	25	29	*1.15*	8	2	*0.27*
106	940	432	0.46	325	52	0.16
108	1550	387	0.25	940	102	0.11
109	1700	340	0.20	910	100	0.11
111	860	154	0.18	780	70	0.09

Ab N = γ-antibody nitrogen in 1 ml. absorbed serum.

S^{35} = counts/minute in Ab N.

Specific activity = specific activity as counts/minute/γ antibody N.

Ag inj. = antigen injected

Only No. 6-97 was reinjected immediately after the day 11 bleeding; both Nos. 6-93 and 6-97 were reinjected immediately after the day 102 bleeding.

— indicates no measurement.

received no antigen so the serum samples obtained subsequently gave a measure of the normal decline of antibody in the absence of a renewed antigenic stimulus. Again antibody in No. 6-97 was removed from the circulation and the first measureable amount of antibody appeared on day 2 after antigen was reinjected. At 5 to 8 days later the titer again reached a maximum though some-

TABLE II

Specific Activity of Antibody in the Serums of Nos. 9-2 and 9-7 before and after the Reinjection of BSA

Time	No. 9-7			No. 9-2		
	Ab N	S^{35}	Specific activity	Ab N	S^{35}	Specific activity
days						
0	1100	0	0	1250	0	0
2⅛	880	8,536	9.7	1180	21,240	18.0
3	915	6,405	7.0	1250	19,375	15.5
4	1080	11,340	10.5	1340	22,780	17.0
5	1050	9,030	8.6	1460	18,396	12.6
6	1040	7,904	7.6	1350	15,525	11.5
16	720	5,256	7.3	780	5,304	6.8
30	410	2,132	5.2	285	969	3.4
51	160	416	2.6	92	97	1.05
55	125	263	2.1	74	56	0.75
56	120	240	2.0	55	40	0.72
Ag → inj.						
57⅓	0			5	7	*1.35*
58	10	29	*2.9*	23	14	0.60
59	130	81	0.62	225	83	0.37
60	380	163	0.43	1200	—	—
61	620	223	0.36	2100	672	0.32
62	720	238	0.33	2300	690	0.30
63	790	245	0.31	1850	555	0.30
65	1040	302	0.29	1700	510	0.30
66	1120	302	0.27	1450	435	0.30
67	1060	286	0.27	1400	420	0.30
292	53	12	0.22	0		
Ag → inj.						
295¼	9	23	*2.59*	0		
296¾	115	31	0.27	21*	65	*3.08*
297	524	39	0.07			
298	1153	16	0.01			
301	2115	155	0.07			
304	1768	210	0.11			
308	718	90	0.12			
311	719	86	0.12			

Ab N = γ-antibody nitrogen in 1 ml. absorbed serum.

S^{35} = counts/minute in Ab N.

Specific activity = specific activity as counts/minute/γ antibody N.

Ag inj. = antigen injected.

* Died after bleeding.

what lower titer than previously. The specific activity of the antibody in the reinjected animal at first exceeded that in the control, then decreased to a level about ½ that of the control after which both showed the same gradual but constant decline of specific activity. At 102 days both animals received an injection of 1 ml. of 1 per cent BSA; at 4 to 7 days after this reinjection of antigen the titer of both animals reached a higher maximum than previously and was particularly higher for No. 6-93 which had been serving as a control

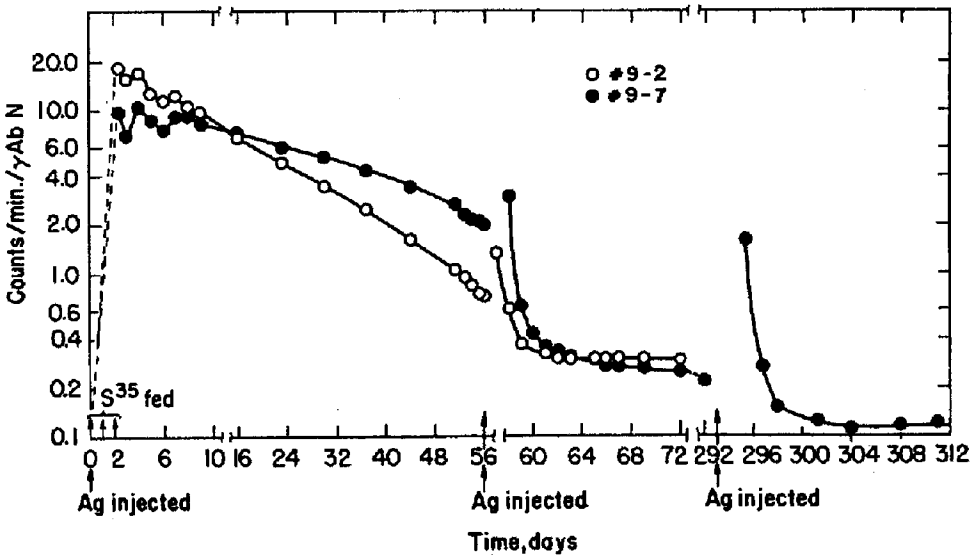

FIG. 2. The specific activity of circulating antibody in hyperimmune rabbits fed S^{35}-labelled yeast cells at days 0, 1, and 2. The last in a series of BSA injections was made simultaneously with the initial feeding of yeast cells at 0 time. No further injection of BSA was made until day 56 when both Nos. 9-2 and 9-7 were again reinjected. The specific activity of antibody is indicated for several bleedings from 57⅛ to 72 days. At day 293, both Nos. 9-2 and 9-7 were reinjected but No. 9-2 died at such an early time after the reinjection that data are insufficient to continue the curve for this animal.

with no injection of antigen for a period of time, exceeding 3 months. With both animals the first precipitable antibody to be measured after the reinjection of antigen was of higher specific activity than that circulating just prior to the renewed antigenic stimulus. The large rise in titer of antibody indicated much new production but the reduction in specific activity was not proportional to the increase in antibody.

Antigen Reinjected at 56 and 293 Days.—

Two other rabbits (Nos. 9-2 and 9-7) received 7 intravenous injections, each consisting of 2 ml. of 1 per cent BSA. After 1 week of rest, 6 injections were given, each as 1 ml. of 1

per cent BSA. After 1 week of rest, immunization was resumed at the lower dose level of ½ ml. of 1 per cent BSA. Three days after the 4th of these injections the animals were bled to obtain a few milliliters of serum for antibody titration. Immediately after the bleeding, 1 ml. of 2.5 per cent BSA was given, followed by a feeding of S^{35} yeast suspension. Two additional yeast feedings followed at 24 and 48 hours from the first. The dose of yeast cells and radioactivity were similar to those in the previous experiment.

A bleeding was made at 6 hours after the 3rd feeding of yeast and at intervals thereafter to determine the antibody in terms of both nitrogen and radioactivity (Table II).

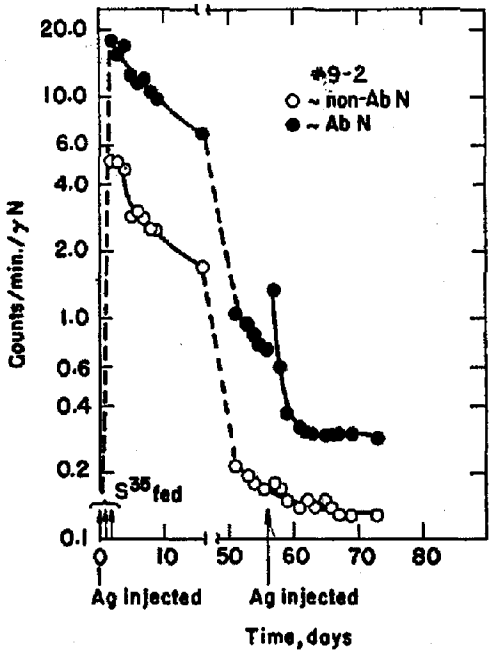

FIG. 3. Comparative specific activity of non-antibody protein nitrogen and antibody protein nitrogen in the serum of rabbit 9-2. S^{35}-labelled yeast cells were fed at 0, 1, and 2 days. BSA was injected at 0 time as the last in a series of injections, and reinjected at 56 days. As in Fig. 2, the few data obtained after the reinjection at 293 days are not included.

From these data the specific activity was calculated and plotted (Fig. 2). Prior to tests with BSA to determine antibody content, all serums were absorbed twice with a pneumococcus SSSII precipitate in an amount of 100 gamma antibody nitrogen per ml. serum. At 56 days, from the last injection of antigen (and also from the time when yeast protein was first fed), the animals were bled in order to obtain a specific activity determination of the serum; then each received 1 ml. of 2.5 per cent BSA. Several bleedings for small amounts of serum were made at intervals during the 1st day after the injection.

All these serums were negative for the presence of specific antibody until the 31 hour bleeding when 1 animal, No. 9-2, had a small amount of precipitating antibody; this antibody had a specific activity greater than that circulating immediately before the reinjection of antigen. The same result, *i.e.* antibody of

high specific activity was likewise found in the serum of No. 9-7, but at the later bleeding time of 48 hours after antigen was reinjected. Therefore the titer rose rapidly to obtain a maximum in No. 9-2 at 5 to 7 days, in No. 9-7, at 9 to 11 days. After the maximal titer was reached the specific activity remained relatively constant for a few days, though at a lower level than prior to the antigen reinjection. No bleedings were made for several months; then at 292 days both animals were bled and the serum was tested interfacially for the

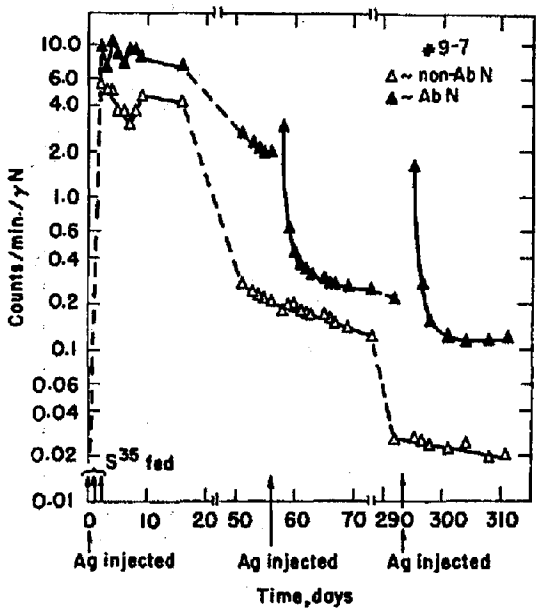

FIG. 4. Comparative specific activity of non-antibody protein nitrogen and antibody protein nitrogen in the serum of No. 9-7. S^{35}-labelled yeast cells were fed at 0, 1, and 2 days. BSA was injected at 0 time as the last in a series of injections and reinjected at 56 and 293 days. During the time after reinjection of antigen, several bleedings were made to follow the levels of specific activity in both non-antibody and antibody protein nitrogen.

presence of antibody. Rabbit 9-7 alone showed a positive test for precipitating antibody at this very long interval of time after antigenic stimulus. At day 293, 1 ml. of 1 per cent BSA was again injected into Nos. 9-2 and 9-7 and frequent small bleedings of No. 9-7 were made at 2 to 5 days and thereafter at longer intervals of time for a few more days. Only 2 bleedings of No. 9-2 were made as the animal died soon after the bleeding on day 296¾. After the injection, the serum became negative for antibody as shown by interfacial testing and remained negative until sometime between 48 and 54 hours for No. 9-7 and 54 to 90 hours for No. 9-2. Beginning with the 54 hour sample of No. 9-7 and the 90 hour sample of No. 9-2 quantitative nitrogen determinations were

made of antibody and the radioactivity was determined in the precipitate at the maximal point of precipitation. The supernates from these maximal points of precipitation were analyzed for nitrogen and also for radioactivity. When these supernates were tested interfacially with antigen and antibody solutions, it was shown that the maximal point of precipitation showed good agreement

TABLE III

Specific Activity of Anti-BSA Antibody Compared in the Presence of Non-Specific and Specific Antigenic Restimulation

Time	Ab N	S^{35}	Specific activity
days			
0	515	0	0
0.3	210	71	0.34
3	178	178	1.00
4	185	634	3.43
7	180	337	1.87
8	150	240	1.60

Ag inj. at day 8 Time	Group I KLH only			Group II BSA + KLH		
	Ab N	S^{35}	Specific activity	Ab N	S^{35}	Specific activity
days						
8.3	110	165	1.50	0		
9	95	143	1.50	41	127	*3.10*
11	73	91	1.25	210	185	.88
13	67	77	1.15	520	385	.74
15	58	57	0.98	575	397	.69
17	51	46	0.90	610	366	.60

Six rabbits received BSA and KLH antigen at day 0. At day 8, three rabbits (group I) received only KLH to test for a non-specific anamnestic response, whereas, the other 3 (group II) received combined BSA and KLH.

Ab N = γ-antibody nitrogen in 1 ml. absorbed serum.

S^{35} = counts/minute in Ab N.

Specific activity = specific activity as counts/minute/γ antibody N.

Ag inj. = antigen injected.

with equivalence. The specific activity of the supernates is used to represent in Figs. 3 and 4 the specific activity of the non-antibody protein nitrogen in serum of Nos. 9-2 and 9-7 rabbits.

The Release of Stored Antibody as a Specific Response to Injection of Antigen.— As a result of the above findings which indicate a reservoir of antibody in addition to the circulating antibody, an investigation was made to determine whether the circulation of such stored material might occur nonspecifically.

Six rabbits received 3 intravenous injections of BSA, each as 2 ml. of 1 per cent solution. After an interval of 1 week, injections were begun with a mixture of BSA and KLH. Six intravenous injections were given, each as 1 ml. containing 10 mg. BSA and 10 mg. KLH in 1 per cent NaCl. Eleven days later, the rabbits received an injection of 10 mg. BSA and 10 mg. KLH as a mixture. Immediately after this injection, each animal was force-fed 15 ml. of a suspension of yeast cells containing 0.35 mc. of S^{35}. All animals were fed yeast cells on the 2 following days, at 24 and 48 hours from the first feeding. Bleedings were made at frequent intervals and the serums were absorbed twice with pneumococcus

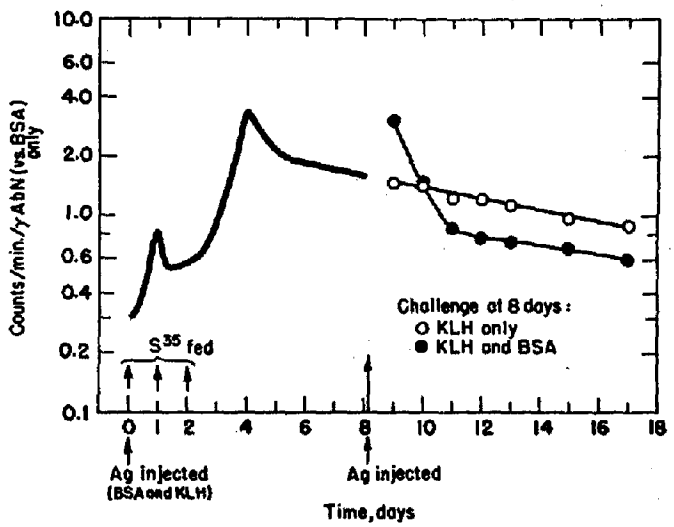

FIG. 5. The specific activity refers to anti-BSA antibody *only*. Six rabbits received the last in a series of injections of combined BSA and KLH simultaneously with an initial feeding of S^{35} yeast cells at day 0. Yeast cells were fed again on days 1 and 2. No further injections of antigen were made until day 8 when three rabbits received KLH and the other three received both KLH and BSA. The curve O refers to the group of 3 rabbits receiving only KLH at day 8 and serves as a control to detect any effects of non-specific antigenic stimulation on the anti-BSA titer. Curve ● is for the group of three rabbits receiving both BSA and KLH.

SSSII precipitates in an amount of 100 gamma N per ml. serum. At 8 days from the simultaneous injection of both antigens and the first feeding of yeast cells, 3 rabbits again received an injection of KLH-BSA and the remaining 3 rabbits were injected with KLH alone. Bleedings were made and the serums were tested for antibody to both antigens. Nitrogen and specific activity determinations for the anti-BSA antibody are pertinent in answering the question which was raised so these data are given in Table III, omitting the data for anti-KLH antibody.

In Fig. 5 a single curve represents the data for the composite group of animals from 0 to 8 days. After the bleeding on day 8, the group was divided. Beginning with the bleeding on day 9 two curves are drawn, 1 curve for animals which received KLH at 8 days and the other for animals injected with both KLH and

BSA as a mixture at 8 days. The results in Table III and Fig. 5 provide evidence that the anamnestic response represents a specific immune mechanism and involves a reservoir of stored antibody.

DISCUSSION

After the reinjection of antigen and as soon as one can measure antibody, the curve of specific activity is already coming down as shown in the figures. It appears thus, that the specific activity of antibody was probably always higher than indicated. This was in fact confirmed by 3 other animals which were used in the same experiments for which results have been plotted in the figures. These particular rabbits were accidentally killed during the course of very frequent bleedings after the reinjection of antigen. However, they did survive sufficiently long to show a specific activity of antibody even higher than given in the figures, with a value more similar to the specific activity obtained when antibody became initially labelled after the feeding of S^{35} yeast cells.

Antibody had higher specific activity than the other serum proteins as was indicated in Figs. 3 and 4. Similar labelling of serum proteins was obtained in all the other animals which were fed S^{35} yeast cells simultaneously with the last in a series of antigen injections. Since a fractionation of the non-antibody proteins was not carried out, it is of course possible that some other form of serum protein may equal or even exceed the antibody protein in specific activity. However, if such a protein were formed, its dilution in a body pool would in all probability diminish the specific activity of the reincorporated protein to a level much lower than that observed for the antibody which is detected early after the reinjection of antigen. Furthermore, the observations of others (5) establish no protein precursor for antibody. Any reincorporation of the labelled amino acids must occur from an amino acid pool, there being no evidence for protein synthesis by significant reincorporation from a peptide pool.

With reincorporation ruled out as a possible explanation for the increase in specific activity of antibody circulating after the reinjection of antigen, the evidence is strong that the antibody persisted in the tissues. The manner in which stabilization of antibody in tissues is maintained requires further investigation but our past experiments which demonstrated long persistence of antigen in liver tissue (6) thus confirming McMaster's earlier work (6 *a*), suggests a possible mechanism. Electrophoretic diagrams of a soluble extract of perfused immune liver tissue demonstrated the presence of a component, not present in normal liver tissue, which had a mobility similar to that of gamma globulin. In the region of this slow electrophoretic component both antigen and antibody could be detected by a serological technique, utilizing free-boundary electrophoresis (7). This is evidence for antigen-antibody complexes existing in liver tissue. Although Miller and Bale (8) concluded from their studies that gamma globulin was not formed in the rat liver, this problem

certainly needs more thorough investigation. For instance, species differences, with regard to antibody production, argue against generalization. There is some indication that all gamma globulin is antibody (9) but it may only be a hyperimmune animal which forms a significant amount of antibody in the liver (10).

The role of the liver is believed to be of importance for the level of circulating antibody, if only in an indirect manner, because of the various correlations which may be drawn between antigen persistence in the liver and circulating antibody. The quantity of antigen persisting was found to be related to circulating antibody (7, 11). Persisting antigen was demonstrated as an RNA complex (6), and during immunization, antigen became associated with tissue components of lower electrophoretic mobility (7). The change in complexing of antigen with liver components of different electrophoretic mobility may indicate only a fragmentation of antigen but it is possible that the different electrophoretic components are composed of antibody of different combining strength. A common observation is that the combining strength of antibody appears to increase during the later stages of immunization which may be correlated with a stronger complexing with antigen in the tissue sites.

The immune paralysis produced by pneumococcus polysaccharide is more probably an example of antigen-antibody complexes persisting in tissues rather than an actual curtailment of antibody production (12). Some such complexing probably always occurs with smaller amounts of any antigenic material.

From the present findings it may be postulated that different antigen fragments are complexed with antibody of varying specific activity. Although there may be equilibration of most antibody molecules, whether in vascular or extravascular pools, some species of antibody molecules appear to exist apart from these equilibrated pools. Such antibody is probably combined with antigen in the tissues. In the case of a high degree of anamnestic reaction, the new production of antibody causes a much reduced level of antibody specific activity as compared with the pre-injection level; however, the failure to assume a lower than observed level of specific activity is evidence that there is a reservoir of antibody of rather high specific activity which is being carried into the circulation. It is obvious that this antibody must have been firmly complexed with antigen and/or the tissues and was not equilibrated with the vascular pool. The evidence for stabilized antibody is thus apparent both in the high specific activity of the small quantity of antibody appearing early after the reinjection of antigen and also in the maintenance of a rather constant specific activity while a very high level of anamnestic response is being attained.

SUMMARY

When rabbits were fed S^{35}-labelled amino acids and simultaneously injected with antigen at the peak of antibody production, the circulating antibody

became rapidly labelled within a few hours with the isotope. The specific radioactivity of antibody was measured as antibody was allowed to decline in the absence of antigenic stimulation. At various times, in different animals, antigen was reinjected and circulating antibody was measured for specific radioactivity. The initial antibody which appeared after the antigenic stimulus always had a higher specific activity than antibody circulating just prior to the reinjection. The appearance of antibody of higher specific activity was demonstrated to be a specific response to the antigen which was reinjected. It was concluded that there is a reservoir of antibody which is stabilized in tissue and which is not in equilibrium with that in circulation. A mechanism for this stabilization is suggested and discussed from previous investigations demonstrating the long retention of antigen in liver tissue.

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